THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 47, Issue of November 21, pp. 46549–46555, 2003

Printed in U.S.A.

A Novel Redox Mechanism for the Glutathione-dependent Reversible Uptake of a Fungal Toxin in Cells*

Received for publication, May 8, 2003, and in revised form, August 21, 2003
Published, JBC Papers in Press, August 28, 2003, DOI 10.1074/jbc.M304825200

Paul H. Bernardo‡, Nicola Brasch§¶, Christina L. L. Chai‡, and Paul Waring‡‖

From the ‡Centre for the Study of Bioactive Molecules, Department of Chemistry, The Faculties, Australian National University, Canberra 0200, Australia and §Research School of Chemistry, Australian National University, Canberra 0200, Australia

The fungal metabolite gliotoxin is characterized by an internal disulfide bridge and can exist in either disulfide or dithiol forms. Gliotoxin and other members of the epipolythiodioxopiperazine class of toxins have immunosuppressive properties and have been implicated in human and animal mycotoxicoses. The bridged disulfide moiety is thought to be generally essential for biological activity. Here we show that only the natural (oxidized) form of gliotoxin is actively concentrated in a cell line in a glutathione-dependent manner. Intracellular levels of the toxin can be up to 1500-fold greater than the applied concentration, and toxin in the cells exists almost exclusively in the reduced form. A simple model of toxin entry followed by reduction to the cell-impermeant dithiol explains active uptake, cell density dependence of EC_{50} values and predicts a value for the maximum concentration of toxin at limiting cell density in agreement with the experiment. Oxidation of the intracellular toxin results in rapid efflux from the cell that also occurs when glutathione levels fall following induction of apoptotic cell death by the toxin. This mechanism allows for minimal production of the toxin while enabling maximal intracellular concentration and thus maximal efficacy of killing in a competitor organism initially present at low cell density. The toxin effluxes from the apoptotic cell exclusively in the oxidized form and can further enter and kill neighboring cells, thus acting in a pseudocatalytic way.

Secondary metabolites of fungal origin are invariably selected for their cytotoxic effects on unicellular competitors and, as part of chemical defense mechanisms (1, 2), are expected to be optimized by selective pressures for cell killing, which may include mechanisms for entry and accumulation in cells. The epipolythiodioxopiperazine (ETP)[1] class of metabolites, which includes gliotoxin (structure **I** in Fig. 1), is characterized by an internal disulfide bridge known to be essential for activity (3, 4). The toxin can exist in either disulfide (**I**) or dithiol form (**II**), depending on the reducing or oxidizing environment.

Gliotoxin has been implicated in human and animal mycotoxicoses (5–7), has immunosuppressive activity (8), and has been used as a tool to inhibit NF-κB (9). Gliotoxin acts as a redox active toxin and in cell-free experiments has been shown to form mixed disulfides with accessible thiol residues on proteins or induce further oxidative modification. Thus, gliotoxin induces internal disulfide formation in proximal cysteine residues in creatine kinase (10) and forms stable mixed disulfides with cysteine residues on alcohol dehydrogenase (11). The easily reduced disulfide bridge across the diketopiperazine ring defines the class and is essential for almost all biological activity of the toxins and has lent support to the idea that it is the disulfide form (**I**) that is the active moiety. Nevertheless, evidence has been presented that the dithiol form may undergo intracellular redox cycling, which may contribute to toxicity (12, 13). Gliotoxin has long been known to display a so called "mass effect" or an increased efficacy of cell killing as cell density decreases (14, 15). Here we show that only the natural (oxidized) form of gliotoxin is reversibly concentrated in a murine cell line. Uptake and cell killing is dependent on intracellular glutathione levels. Active concentration in cells is dependent on an external oxidizing environment and the normal intracellular reducing milieu maintained by glutathione. A simple model of toxin entry followed by rapid reduction to the cell-impermeant dithiol form explains active uptake and allows for minimal production of the toxin while enabling maximal concentration in a putative competitor organism that would be expected to be initially present at low cell density. Decreased intracellular glutathione following induction of apoptosis by gliotoxin results in release of active toxin, which is then made available for further cell killing.

EXPERIMENTAL PROCEDURES

Chemicals—^{35}S-Labeled gliotoxin was prepared and purified as described (16). Gliotoxin was prepared as a stock solution in absolute ethanol at 1 mg/ml. All other chemicals were purchased from Sigma.

Uptake of Labeled Gliotoxin—P388D1 cells were obtained from the ATCC. Cells were treated at various times and cell densities with gliotoxin of known specific activity. Cells were pelleted and washed once, the cell pellet was lysed in 5% SDS, and radioactivity was counted using a Beckman LS6500 scintillation counter. Counts/min was converted to mass of toxin using a standard curve, which was generated weekly. Initial rates of uptake were determined following a 20-s treatment with labeled toxin and rapid (5–10-s) pelleting of cells in a microcentrifuge without washing. In these experiments, extracellular gliotoxin could be allowed for, given the known concentration in extracellular fluid and the estimated volume of cell pellet (cells plus excess extracellular fluid amounted to less than 20 μl). This amounted to less than 2% of total cell-associated toxin.

Volume of P388D1 Cells—Volume was determined by measurement of cell diameter on 15 cells chosen at random using a Bio-Rad Radiant 2000 confocal microscope. Under these conditions and using Z series, the cells were shown to be almost completely spherical. Intracellular concentrations of toxin were calculated on the basis of uniform distribution in cells.

HPLC Analysis—HPLC was carried out using Beckman System Gold equipment. Cell pellets were lysed using cold 5% trichloroacetic acid

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This paper is dedicated to the memory of Dr. Joanne Beaver.

¶ Present address: Dept. of Chemistry, Kent State University, Kent, OH 44224.

‖ To whom correspondence should be addressed. Tel.: 62-261252022; E-mail: Paul.Waring@anu.edu.au.

[1] The abbreviations used are: ETP, epipolythiodioxopiperazine; HPLC, high pressure liquid chromatography.

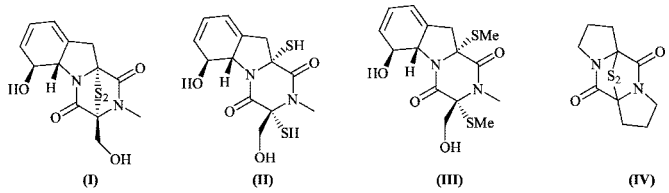


FIG. 1. **The structure of gliotoxin (I), reduced gliotoxin (II), the bismethylthioether of gliotoxin (III), and the simple diprolyl ETP compound (IV).**

degassed to inhibit subsequent oxidation of reduced toxin. Toxin was separated on a Phenomenex Hypersil C18 reverse phase column, eluted with a gradient of 5–40% aqueous acetonitrile, and detected by UV at 270 nm. In some cases, radiolabeled toxin from cells was analyzed by HPLC, and fractions were collected. These experiments showed that radiolabel was associated only with either reduced or oxidized toxin and that no other radiolabeled metabolites were present. In particular, we could detect no additional peaks that might correspond to the mono mixed disulfide between toxin and glutathione either inside the cell or in extracellular medium within the first 30 min of treatment.

Flow Cytometric Analysis—Fluorescence-activated cell sorting analysis was carried out on a BD LSR Benchtop Flow Cytometer with cells at 0.5–1 $\times$ 10^6/ml. Apoptosis quantification using propidium iodide (17) and monochlorobimane staining (18) were determined according to published procedures.

Reduction of Gliotoxin by Glutathione—The rate of reduction of gliotoxin by glutathione was performed on an Applied Photophysics SX.18MV stopped flow reaction analyzer at 25 °C, monitoring an absorbance increase of reduced toxin at 250 nm. HPLC analysis showed that the only product of this reaction was the reduced toxin.

*Synthesis of the Simple Diprolyl ETP Toxin (**IV**)*—This compound was synthesized according to the procedure in the literature (19).

Antiproliferative Effects—The effects of gliotoxin and analogues on cell growth were measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (20). Toxin was diluted 2-fold down rows in 96-well plates, and cells at varying initial densities were added at time zero. Final cell numbers were assessed at 3–4 days as described.

Cellular Glutathione—Intracellular glutathione and oxidized glutathione were determined using the OxisResearch GSH/GSSG-412™ assay kit, which uses the modified Tietze method to assess both the reduced and oxidized forms of glutathione.

RESULTS

Active Uptake of Gliotoxin Requires the Bridged Disulfide Bond—We have previously reported that gliotoxin actively accumulates in a variety of cell types including human and murine cell lines as well as murine lymphocytes and that up to 40% of the total mass of toxin added to a cell suspension becomes cell-associated within 5–10 min (16). These experiments were carried out with cell densities of 1 $\times$ 10^6/ml. Because active uptake and extrusion of toxins from cells has important implications for both efficacy of action and development of resistance to chemotherapeutic agents (21), we decided to study the mechanism of this active uptake using the P388D1 murine tumor cell line. Treatment of P388D1 cells at 1 $\times$ 10^6/ml with gliotoxin resulted in a 300-fold accumulation of toxin (Fig. 2*A*), with maximal levels by 15–20 min. Almost 50% of the maximally accumulated toxin is present within 2 min. Table I shows typical experimentally determined intracellular concentrations for differing applied toxin concentrations. The table also shows predicted intracellular concentrations based on the proposed uptake mechanism (see below). Key to understanding the uptake mechanism was the observation that the biologically inactive bismethylthioether (22) (**III**) is not significantly concentrated in cells. In addition, extracellular agents known to reduce gliotoxin to the dithiol form also abrogate uptake (Fig. 2, *B* and *C*), indicating that the disulfide bridge is required for active accumulation of the toxin. We initially explored known possible mechanisms that may facilitate active uptake of small molecules. Initial rates of uptake showed saturation kinetics and were independent of Na^+ (Fig. 2*D*) and

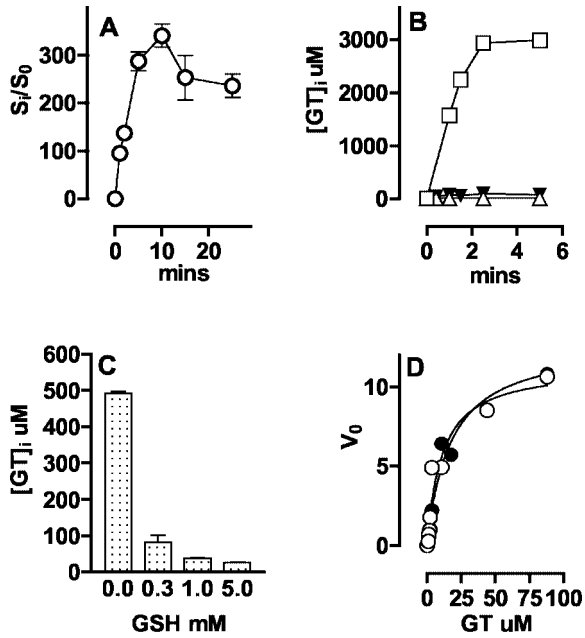


FIG. 2. *A*, intracellular concentration of gliotoxin in P388D1 cells treated with 1 μM gliotoxin. S_i/S_o is the ratio of intracellular to extracellular toxin. *B*, intracellular concentrations in P388D1 cells treated with 10 μM gliotoxin (□), reduced gliotoxin (▼), and the bismethythioether of gliotoxin (△). *C*, extracellular glutathione inhibits uptake of 3 μM gliotoxin into P388D1 cells. Toxin levels were measured 35 min after treatment. *D*, saturation kinetics of initial rates of uptake of gliotoxin in the presence (○) or absence (●) of Na^+. V_0 is given in nmol of toxin/10^7 cells/min.

TABLE I
Intracellular Gliotoxin Concentration in P388 Cells

$[GT]_{Applied}$ is the concentration applied at $t = 0$; $[GT]_i$ is the experimentally determined intracellular levels, and $[GT]_{Calc}$ is the value determined using Equation 1 using $P = 1151$ and $V_C = 0.0013$. Toxin levels were measured 15 min after treatment. Cells were 1 $\times$ 10^6/ml.

$[GT]_{Applied}$	$[GT]_i$	$[GT]_{Calc}$
	μM	
0.1	25 ± 3	45.2
0.3	115 ± 10	135
1.0	277 ± 30	452
3.0	1020 ± 90	1360

unaffected by inhibitors of glucose transport or of amino acid uptake such as BCH (data not shown). Initial rates also showed a pH maximum at 7.0 with a fall in V_0 at higher pH (data not shown). These data indicate that neither a sodium gradient (sugars) nor a proton gradient (peptides) (23, 24) were required for active accumulation in cells.

Active Uptake of Gliotoxin Is Glutathione-dependent—We then sought alternative mechanisms that might account for active accumulation of only the oxidized form of the toxin. Uptake of gliotoxin was abrogated when cells were depleted of glutathione by diethyl maleimide or diamide. Inhibition of uptake parallels loss in cellular glutathione (Fig. 3, *A* and *B*). The effect of diamide is reversible, and the ability to concentrate gliotoxin in cells is restored when they are washed free of diamide and placed back into fresh medium (Fig. 3*C*). This is also true of the initial rate of uptake (Fig. 3*D*). This reversibility of uptake is due to rapid reduction of oxidized glutathione by glutathione reductase once the oxidative stress induced by diamide is removed. 1,3-Bis(2-chloroethyl)-1-nitrosourea, an inhibitor of GSH reductase, inhibits recovery of GSH (Fig. 3*E*). The observed increase in glutathione over controls following diamide treatment and washing (mechanism unknown) is consistent with increased uptake as in Fig. 3*C*. The above data

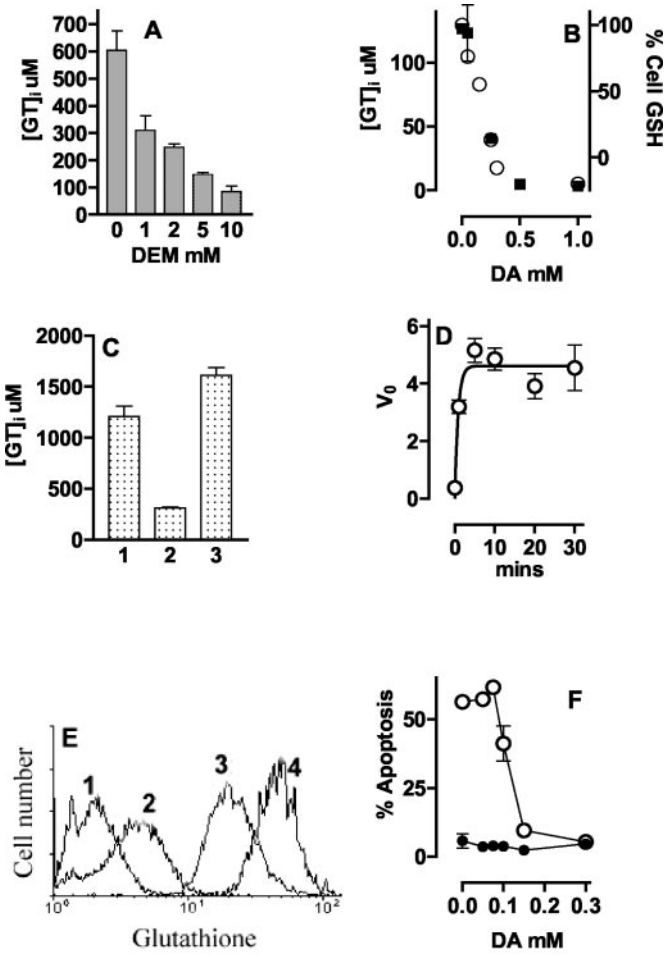


FIG. 3. *A*, depletion of P388D1 cells of glutathione by diethyl maleimide (*DEM*) inhibits uptake of 3 μM gliotoxin. Toxin levels were measured at 35 min after treatment. *B*, dose-dependent abrogation of uptake of 0.3 μM gliotoxin by diamide (*DA*) 10 min after treatment. ○, gliotoxin levels in cell; ■, percentage change in monochlorobimane fluorescence equivalent to percentage decrease in glutathione levels. Similar results were found for other concentrations of toxin. *C*, abrogation of uptake by diamide is reversible. *1*, intracellular toxin after 15-min treatment with 3 μM gliotoxin; *2*, the same experiment after treatment of cells with 300 μM diamide for 5 min; *3*, the same batch of cells in *2* washed and placed back in fresh culture media for 10 min and treated with labeled toxin for 15 min. *D*, recovery of initial rates of uptake after diamide treatment. V_0 was measured with 3 μM gliotoxin at varying times after P388D1 cells were treated with 300 μM diamide and placed back into fresh culture medium. *E*, recovery of GSH in P388D1 cells following diamide treatment is inhibited by 1,3-bis(2-chloroethyl)-1-nitrosourea. *1*, cells treated with 300 μM diamide for 5 min followed by MCB staining (glutathione-depleted); *2*, cells pretreated with 20 μM 1,3-bis(2-chloroethyl)-1-nitrosourea for 30 min followed by 300 μM diamide, for 10 min, placed back into fresh medium, and then stained with MCB (glutathione reductase-inhibited and glutathione-depleted, no recovery of glutathione); *3*, normal cells stained with MCB (normal glutathione levels); *4*, cells treated with 300 μM diamide for 10 min, washed, placed back into fresh medium, then stained with MCB (recovery of glutathione) (all data gated on live cells only). *F*, diamide inhibits gliotoxin-induced apoptotic cell death in P388D1 cells. ●, cells treated with diamide only for 10 min, washed, and placed into fresh medium; ○, cells treated with diamide for 10 min followed by 3 μM toxin for 5 min, washed, and placed in fresh medium. Apoptosis was assessed at 24 h.

clearly demonstrate a requirement for intracellular glutathione in the accumulation of gliotoxin in P388 cells. In order to show that uptake was necessary for apoptotic cell killing, we transiently depleted cells of glutathione using diamide and treated them for a short period with unlabeled gliotoxin. Treated and control cells were then washed and placed back into fresh media and harvested 24 h later. Apoptotic cell death induced by gliotoxin was also dependent on intracellular glutathione and was abrogated by diamide (Fig. 3*F*). Transient depletion of glutathione by diamide had no long term effect on cell viability. It was possible that the driving force for toxin entry was provided by the large concentration gradient of glutathione across the cell membrane in the opposite direction. This would result in exchange of toxin molecules for glutathione as occurs in the OATP1 type exchanger. We eliminated this possibility by first showing that initial uptakes were unaffected by rifampicin, a known inhibitor of OATP1, and that there was no detectable glutathione effluxing from cells over the first 20 min of uptake of gliotoxin.

Intracellular Gliotoxin Is Rapidly Reduced to the Dithiol Form—We then examined the intracellular nature of the accumulated toxin using HPLC. Intracellular gliotoxin was present predominantly in the reduced form (structure **II**; Fig. 1). By 2 min, the shortest sampling time possible for HPLC analysis, or at the time of maximum gliotoxin load, most of the toxin was reduced (Fig. 4, *A–C*). Reduced gliotoxin was unambiguously confirmed by retention times and the demonstration that, as expected, reduced gliotoxin from cell lysates subsequently oxidized back to **I** (Fig. 4*D*).

Given that the small amount of oxidized toxin seen in Fig. 4*C*, for example, could arise from rapid autoxidation of a small amount of reduced toxin once cells are lysed, it is clear that almost all of the toxin in the cell is in the reduced form. Accumulation of toxin as the reduced form in the cell suggests a mechanism of rapid metabolic conversion of the toxin driving accumulation down a concentration gradient until equilibrium is reached. This would require both rapid conversion to reduced toxin and an equilibrium position favoring the reduced form. We have previously reported the equilibrium constant between gliotoxin and glutathione as 1200 M^{-1} (25). This strongly favors the reduced form (**II**) in the presence of excess glutathione. Initial uptake of toxin is relatively fast, and, if our initial proposal is correct, would require rapid reduction of toxin by glutathione once it entered the cell. Initial experiments using conventional spectroscopy showed that the rate of reduction of gliotoxin by glutathione was very rapid, and stopped flow techniques were required to show that the apparent first order rate constant at 25 °C and pH 7.0 for formation of reduced gliotoxin from 50 μM gliotoxin was 37.4 s^{-1} in the presence of 5 mM glutathione. The latter is typical of intracellular glutathione levels. This corresponds to a half-life of 18.5 ms, more than rapid enough to account for the initial rates of uptake of gliotoxin if reduction is driving accumulation. Table II shows second order rate constants for reduction of gliotoxin at differing pH values. The apparent rates were first order in GSH and gliotoxin, and the second order rate constants increase ~10-fold for each rise in pH, indicating that reduction occurs via attack of the thiolate of glutathione on the disulfide of the toxin as expected by analogy with similar mechanisms for reduction of somatostatin by glutathione (26). Importantly, the presence of a mixed disulfide intermediate between toxin and glutathione could not be detected using HPLC or mass spectrometry as mentioned earlier for toxin-treated cells. Only the dithiol form (**II**) is detectable in the presence of glutathione, indicating that any putative mixed disulfide intermediate rapidly reacts with a second molecule of GSH to produce **II** and GSSG (26).

A Simple Model for Uptake of Gliotoxin—These results led us to propose a simple redox mechanism that accounts for accumulation of the toxin in cells involving entry of the toxin into the cell followed by rapid reduction to the cell-impermeant reduced form of the toxin. Data from Table I were generated using 1×10^6 cells/ml. We determined that P388D1 cells have an average volume of 1.3×10^{-9} ml. For 10^6 cells, this corresponds to a total volume of 1.3 μl (*i.e.* a little more than

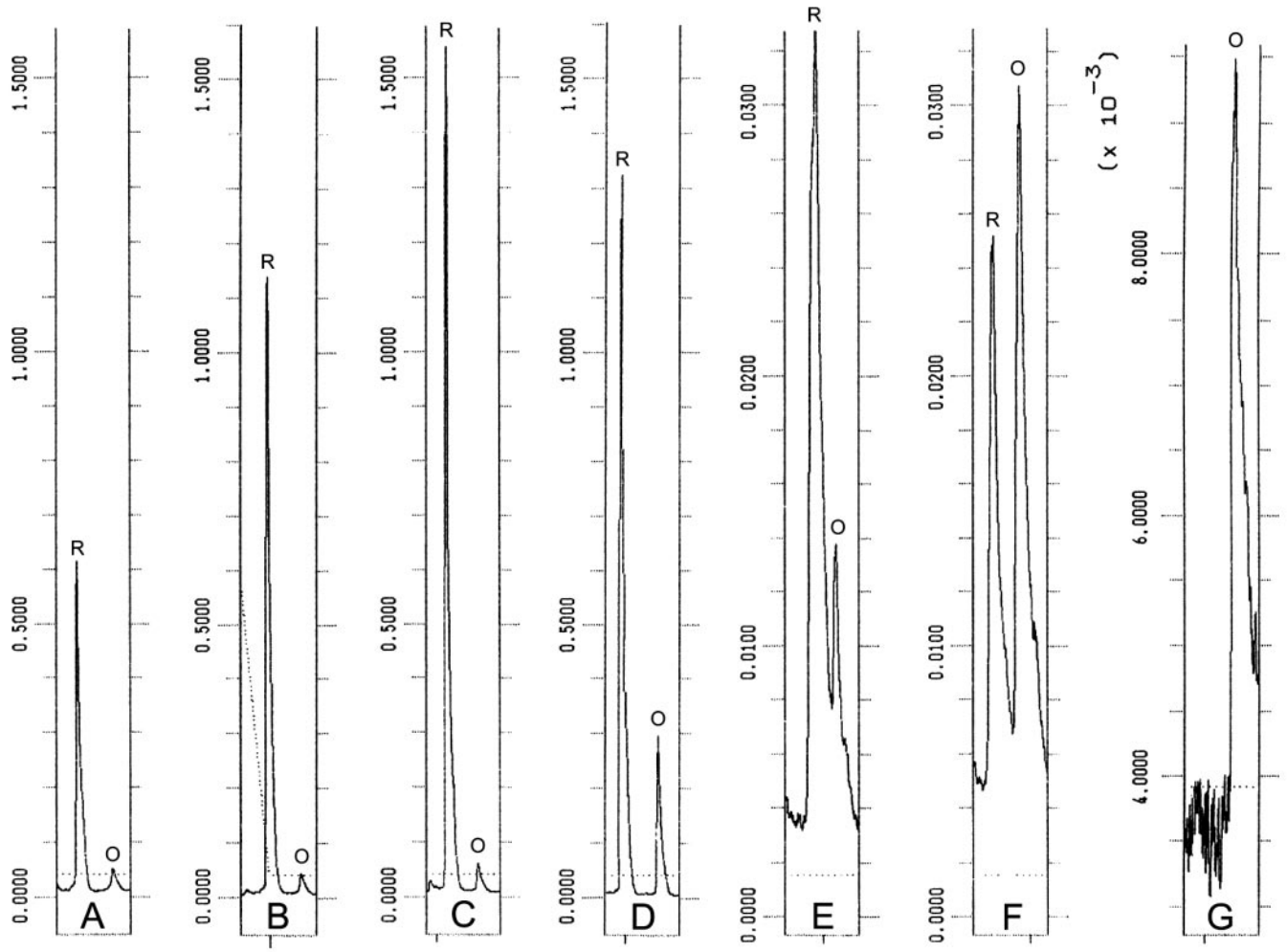
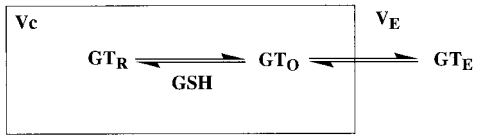


FIG. 4. **HPLC analysis of cellular and extracellular gliotoxin.** *R*, reduced toxin; *O*, oxidized (natural form) toxin. *A–C*, intracellular toxin 2, 5, and 10 min after treatment of cells with 50 μM gliotoxin; *D*, lysate from *C* left at room temperature for 15–20 min; *E*, intracellular diprolyl ETP (structure IV) 10 min after treatment with 50 μM diprolyl ETP under the same conditions as *C*. Note the large difference in scales, indicating that compound **IV** is present at 2% of the level of gliotoxin. *F*, lysate from *E* after 15–20 min at room temperature. *G*, extracellular gliotoxin effluxing from P388D1 cells 2 h after treatment with 10 μM toxin and washed is in the oxidized form only. Compound IV was present in cells at 2% of the level seen with gliotoxin under the same conditions. Intracellular concentration of reduced toxin in *A*, *B*, and *C* was 5070, 9300, and 13,500 μM, respectively, consistent with data in Table I.

TABLE II
Second order rate constants for reduction of gliotoxin by glutathione

Values for $k \times 10^{-3}$ are shown. The second row shows estimated half-lives in milliseconds for the following conditions: 3 μ M gliotoxin, 5 mM glutathione.

pH 8.0	pH 7.0	pH 6.0
72.3 ± 0.9	7.67 ± 0.04	0.74 ± 0.02
(1.92)	(18)	(187)



SCHEME 1. **A simple model for toxin accumulation.**

1000-fold greater than the extracellular volume in a typical experiment). In developing our model, we assume a total combined cellular volume (V_C) of 0.001 liter in 1 liter of extracellular fluid (V_E), corresponding very approximately to the ratio between the total volume of 10^6 cells in 1 ml of medium (*i.e.* we assume that the total volume of the cells can be assumed equivalent to a single volume V_C. At equilibrium, we denote extracellular gliotoxin GT_E, oxidized toxin entering the cell GT_O, and reduced toxin in the cell GT_R, as shown in Scheme 1. The initial concentration of toxin added to the cells we denote $GT_{Applied}$.

The following argument is independent of the route of entry of toxin. Initially for simplicity, we assume that 1 mol of gliotoxin is added to V_E (*i.e.* $GT_{Applied} = 1$ M), and at equilibrium *m* mol of toxin have entered V_C. The following relationships hold: (i) at equilibrium the external toxin concentration is $(1 - m)M$, since $V_E = 1$ liter; (ii) the total intracellular toxin concentration will be m/V_C M (*i.e.* the concentration assessed using radiolabeled toxin, which cannot discriminate reduced from oxidized form of toxin); (iii) $([GT_R] \times [GSSG])/([GT_O] \times [GSH]^2) = 1200$ M^{-1} by definition; (iv) $m/V_C = [GT_O] + [GT_R]$ by definition. We assume (v) the concentration of glutathione does not drop significantly over the time required for toxin accumulation (supported by experimental data; see below); (vi) intracellular reduced gliotoxin, GT_R, does not significantly efflux from cells (the reduced form remains in the cell for at least 20 min; Fig. 2*A*); and (vii) GT_O is in equilibrium with GT_E, and rapid efflux of oxidized gliotoxin following diamide treatment supports this. From (iii) above $[GT_R]/[GT_O] = 1200 \times ([GSH]^2/[GSSG]) = P$, a constant (given point *V* above). Thus, $[GT_R] = P \times [GT_O]$, and thus $m/V_C = [GT_O] + P \times [GT_O]$. However, $[GT_O] = [GT_E] =$

$(1 - m)/V_0$ or $(1 - m)$, since $V_0 = 1$ liter.

Thus, $m/V_C = (1 - m) + P \times (1 - m)$, which can be solved for m, giving the equation, $m = (V_C \times (1 + P))/(1 + V_C + P \times V_C)$ or $m/V_C = (1 + P)/(1 + V_C + P \times V_C)$ for 1 mol of toxin added (*i.e.* 1 M initial concentration).

The following can then generally be easily shown,

$$m/V_C \text{ (or } [GT]_i) = ([GT_{Applied}] \times (1 + P))/(1 + V_C + P \times V_C) \quad \text{(Eq. 1)}$$

where $[GT_{Applied}]$ represents any initial concentration of toxin applied to the cell suspension in appropriate units, and $P = K_{eq} \times ([GSH]^2/[GSSG])$, where K_{eq} is the equilibrium constant between gliotoxin/GSH and reduced gliotoxin/GSSG, and V_C is the *total* volume of cells in unit volume of extracellular fluid. This describes the equilibrium concentration of toxin in the cell.

Thus, m/V_C, which we denote $[GT]_i$ or the *total* intracellular concentration of toxin (reduced plus oxidized form), can be expressed in terms of the applied concentration, the number of cells (equivalent to V_C), the equilibrium constant for reduction of the toxin by glutathione (K_{eq}), and the $[GSH]^2/[GSSG]$ ratio.

Experimental Justification of the Model—In P388 cells, we determined [GSH] to be 4.67 ± 0.67 mM, assuming uniform distribution, and the value of [GSH]/[GSSG] was 198 ± 76. We also note that GSH and GSSG levels in cells treated with up to 10 μM gliotoxin were also determined in this way. Gliotoxin had no effect on either GSH or GSSG levels up to 30 min after treatment with toxin. Using $V_C = 0.0013$ (based on 10^6 cells/ml), Table I shows that calculated intracellular gliotoxin is in remarkable agreement with the experimentally determined values when equilibrium is reached, consistent with the proposed model. The model overestimates $[GT]_i$ probably because of wash out when measuring toxin loads and lack of homogeneity in cellular GSH.

Equation 1 also predicts that there is a linear relationship between applied toxin and $[GT]_i$ and furthermore that the slope of the line should increase with decreasing cell density. This is confirmed in Fig. 5*A*. For a given applied concentration, toxin load will increase as cell numbers (V_C) decrease, approaching a maximal value determined by $(P + 1)$. The most dramatic consequences of this model, however, follow from the prediction that, for a given applied extracellular concentration of toxin, intracellular toxin loads should increase and approach a constant maximum value when cell density approaches a very small number. This is shown in Fig. 5*B*, where the intracellular gliotoxin clearly approaches a value between 1500 and 2000 μM as cell density decreases when cells are treated with 1 μM gliotoxin. Approach to constant intracellular load as V_C approaches zero is consistent with the predictions of Equation 1, which predicts a limiting value of about 1200 μM for Fig. 5*B*.

Efficacy of Cell Killing by Gliotoxin is Cell Density-dependent—Gliotoxin is a potent antiproliferative agent, and nanomolar EC_{50} values have been reported (27). Because of the results shown in Fig. 5*B*, we measured the effectiveness of gliotoxin as an antiproliferative agent against P388D1 cells at various cell densities. The EC_{50} values thus determined show a rapid convergence to a limiting value of under 3 nM as shown in Fig. 5*C*. It is axiomatic that for a toxic molecule of given structure and for a given cell type under defined conditions, there will be a unique average intracellular concentration that will determine its efficacy as determined by EC_{50} values. Equation 1 can be simply reformulated as follows.

$$[GT_{Applied}] = [GT]_i \times (1 + V_C + P \times V_C)/(1 + P) \quad \text{(Eq. 2)}$$

Thus, the magnitude of $[GT]_{Applied}$, which determines the experimental EC_{50} value, required to establish some constant intracellular concentration ($[GT]_i$) will approach a minimal value determined by P as the cell numbers approach zero. This is exactly the behavior shown in Fig. 5*C*, where the EC_{50} value approaches 2–3 nM as cell density approaches zero. Similar behavior is shown by other members of the ETP family (data not shown). This model thus provides a simple explanation for the "mass effect" of ETP toxins, where efficacy of biological effect is dependent on cell density.

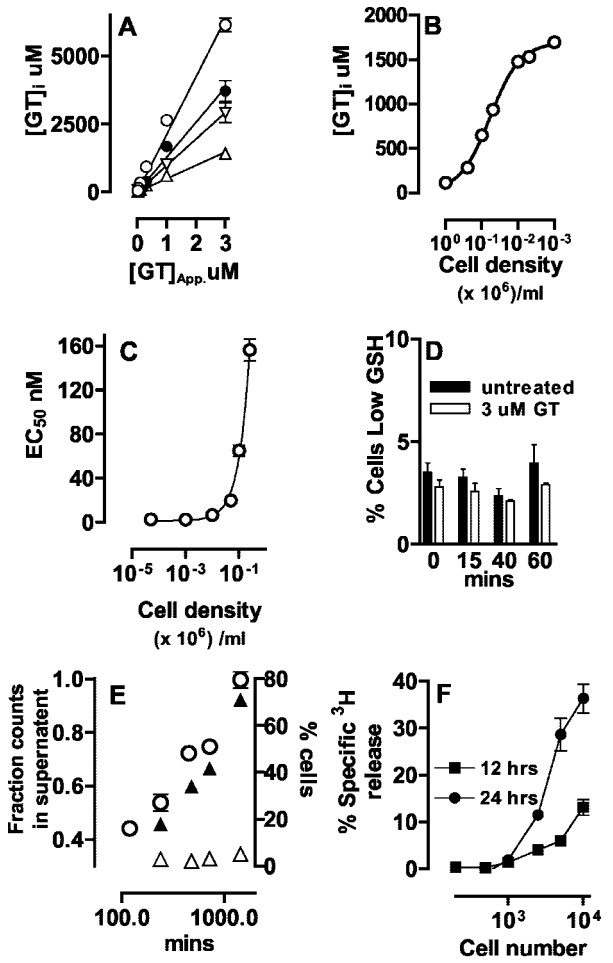


FIG. 5. *A*, relationship between applied toxin concentrations and intracellular toxin load at differing cell densities. △, 1×10^6/ml; ▽, 0.5×10^6/ml; ●, 0.25×10^6/ml; ○, 0.1×10^6/ml. *B*, the intracellular toxin load approaches a maximal limiting value as cell density approaches zero for a given applied concentration of 1 μM after 25 min. *C*, the EC_{50} value for inhibition of proliferation of P388D1 cells by gliotoxin approaches a minimal value of 2–3 nM as cell density approaches zero. *D*, glutathione levels do not fall in the first 60 min after treatment with 3 μM gliotoxin. *E*, decrease in glutathione following apoptosis induced by gliotoxin treatment of P388D1 cells parallels increased levels of extracellular toxin. △, percentage of cells with low GSH in control; ▲, percentage of cells with low GSH treated with 3 μM gliotoxin; ○, released toxin assessed as fraction of total counts/min released to extracellular medium. *F*, gliotoxin released from P388D1 cells undergoing apoptosis can kill bystander cells. 96-Well plates with 20,000 [^{3}H]thymidine-labeled P388D1 cells untreated with toxin per plate were exposed to differing cell numbers of P388D1 cells treated with 10 μM gliotoxin for 10 mins and washed free of all extracellular toxin.

Gliotoxin-induced Glutathione Loss Results in Efflux of Oxidized Toxin—Gliotoxin induces apoptotic death that is associated with a concomitant drop in intracellular glutathione (28). Efflux of glutathione has also been shown to occur in apoptotic cell death triggered by other stimuli (29). In our proposed model, we assume that GSH levels remain constant over the period during which we measure uptake of toxin (*i.e.* 10–15 min). This can occur by rapid regeneration of GSH from GSSG by glutathione reductase and is supported by data in Fig. 3*E*. In

TABLE III
Efflux of gliotoxin from P388D1 cells

Time		Gliotoxin
min		μM
30		0.57 ± 0.15^a
120		1.84 ± 0.20^a
240		2.95 ± 0.30^a
330		3.63 ± 0.50^a
	With DA	Control
	μM	μM
10	1.0 ± 0.1	0.20 ± 0.05^b
30	1.1 ± 0.15	0.36 ± 0.05^b
120	1.1 ± 0.15	0.62 ± 0.07^b

[a] Cells were treated with 10 μM toxin for 10 min, washed, and placed into fresh medium. Oxidized toxin only was detected (see Fig. 4*G*).

[b] Cells were treated with 3 μM toxin for 20 min, washed, and placed into fresh medium and either left untreated or treated with 500 μM diamide at $t = 0$. Less than 5% toxin remains in cells after diamide treatment. All concentrations were measured in extracellular medium using HPLC.

fact, GSH levels do not drop in gliotoxin-treated cells for at least 60 min after treatment (Fig. 5*D*), justifying our assumption in the development of our simple model. This also shows that exposure of cells to gliotoxin does not simply result in GSH loss by simple chemical depletion. We then examined the fate of the accumulated gliotoxin after induction of apoptotic cell death. Cells treated with gliotoxin, washed, and placed back into fresh medium release gliotoxin as the oxidized form as shown in Fig. 4*G*. Table III shows the kinetics of released gliotoxin from otherwise unperturbed cells and shows that diamide-treated cells release gliotoxin much more rapidly after preloading with the toxin. This confirms that the dithiol form does not easily pass through the cell membrane, whereas the oxidized (normal) form can. It also provides evidence that gliotoxin is not held in the cell by covalent interaction with proteins via disulfide bonds. The latter would be released by reducing conditions rather than oxidative ones. In our model, efflux would be hastened by attempts to re-establish equilibria with extracellular toxin if cells were washed and placed into fresh medium. We therefore examined efflux of toxin from unwashed, unperturbed cells (*i.e.* in the presence of equilibrium oxidized toxin). This showed a correlation between apoptosis, loss in glutathione levels, and net loss of toxin from cells. In other words, triggering of apoptosis, which results in GSH loss, also results in loss of oxidized toxin from the cell, because the equilibrium in Scheme 1 is now disturbed. As expected, the toxin that is released remains biologically active (Fig. 5, *E* and *F*). In Fig. 5*E*, the fraction of toxin cell associated following treatment is just less than 50% as assessed by counts in the supernatant (and consistent with Table I). This increases to 100% release by 18 h. Thus, most of the toxin taken into the cell is released and can be "recycled" following induction of apoptotic cell death.

Uptake and Cytotoxicity Is Abrogated in a Simple Analog with Reduced Reduction Potential—Our model suggests that reduction potential is a strong determinant of uptake of gliotoxin and thus of biological activity. A corollary of this is that an ETP toxin with lower reduction potential would be both less active and concentrate in cells less efficiently than the natural product. In our screening of synthetic ETP toxins, we found that the synthetic diprolyl ETP toxin (**IV**) had dramatically reduced biological activity compared with the natural product. Compound **IV** has an EC_{50} value of 0.94 ± 0.15 μM compared with a value for gliotoxin of 0.058 ± 0.01 μM for inhibition of proliferation of P388D1 cells under identical conditions. This represents less than 6% activity of the natural product. Uptake of **IV** also occurs with concomitant reduction to the dithiol form (Fig. 4, *E* and *F*), although its concentration in cells was only 2% of that of the natural product at the same concentration. We determined the equilibrium constant for reduction of **IV** with glutathione as 6.8 ± 0.5 M^{-1}. This makes the value of P some 175-fold less than that for gliotoxin, consistent with both the reduced toxicity and reduced intracellular levels of compound **IV**. The reason for the greatly reduced reduction potential of this synthetic ETP toxin is unknown but may be related to favorable release of strain when in the disulfide form. We are currently examining structural requirements that determine reduction potential in ETP toxins.

DISCUSSION

Much effort is expended on studies of structure-activity relationships in attempts to delineate the pharmacophore in a biologically active molecule. This includes attempts to correlate reduction potentials in redox-active toxins such as quinones to biological activity (30). This type of study often focuses on the intracellular site of action of the toxin rather than considering secondary effects such as the effective intracellular concentration of the toxin, which must contribute to the efficacy of the toxin. In this work, we show that the unique structural features of gliotoxin confer the ability of reversible accumulation in cells by a redox mechanism resulting in very high intracellular concentrations and thus high efficacy in cell killing. Furthermore, examination of an analogue with greatly diminished biological activity confirmed that the redox potential of the toxin determines its intracellular concentration and hence its efficacy. Although the precise mechanism of induction of apoptotic cell death by ETP toxins is unknown, putative targets include thiol-dependent enzymes such as creatine kinase (10), the transcription factor NF-κB (9), Ras farnesylating enzyme (31), and the mitochondria (32, 33). In all instances, the efficacy of the toxin will depend on the effective intracellular concentration of the molecule. This work still leaves open the possibility that either the reduced or oxidized forms are responsible for toxicity once in the cell, since there will be a small but finite quantity of oxidized form available for interaction with proteins. The presence of the reduced toxin in significant amounts will then act as a pool for more of the oxidized form once the equilibrium established by GSH is disturbed. The model does provide an explanation for the observation that activity is only associated with the oxidized form when presented outside of the cell. Design of analogs of gliotoxin with varying reduction potentials to achieve differential intracellular concentrations is under way. It may also be possible to conjugate a second molecule to the ETP skeleton to affect active transport of a second toxin into a cell. This will occur in a glutathione-dependent manner. Understanding the generality of this process may aid in overcoming the problem of drug-resistant cells associated with increased production of glutathione (34).

Selection of gliotoxin by a fungi as part of a chemical defense mechanism against unicellular competitors has obvious advantages. Glutathione has been detected in many healthy single cell organisms (35). Thus, uptake of gliotoxin into cells is facilitated by subversion of a normally protective mechanism to affect intracellular concentrations that are orders of magnitude greater than the extracellular concentration of toxin. Accumulation requires a normal, external oxidative milieu and a strongly reducing intracellular environment. Importantly, no mixed disulfide with glutathione is detectable that would represent a possible detoxification step; instead, the membrane-impermeant dithiol is the only product reversibly formed. This process has a direct parallel in the uptake and subsequent release of Fe(II) from extracellular siderophore bound Fe(III) after transport of the latter into bacterial cells (36). We believe

this is the first example of a naturally occurring toxic secondary metabolite that has exploited the difference in redox state outside and inside the cell to affect reversible intracellular accumulation. The reductant does not have to be limited to glutathione and could include other small molecules such as lipoic acid or coenzyme A. The latter is present in many Gram-positive bacteria that are generally low in glutathione (37). Unicellular competitors would be expected to be initially present at low numbers, and here we show that these conditions would favor maximum accumulation of toxin in healthy cells. Furthermore, data in Fig. 5*A* show that greater incremental increases in intracellular toxin will occur for a given incremental change in applied toxin as the cell density approaches zero, again maximizing efficient use of the secreted toxin. ETP toxins are unique in that the presence of the internal disulfide bond allows reduction to be easily reversed under appropriate conditions, reforming the oxidized (natural) form of the toxin, which then effluxes from the cell. This suggests an elegant mechanism involving rapid uptake and killing of cells followed by recycling toxin from apoptotic cells to enter and kill bystander cells, maximizing the efficiency of this naturally occurring cytotoxic agent. Release of toxin from cells triggered to undergo apoptosis could minimize further covalent interaction with protein that would be wasteful and unproductive. Although our model, which describes the equilibrium levels of toxin in the cell, is independent of the route of entry through the plasma membrane, saturation kinetics suggests specific facilitated transport of the oxidized form rather than diffusion. Lack of diffusion of high concentrations of reduced toxin from the cell also supports this. We are currently investigating a possible transport mechanism involved in carrying the toxin across the plasma membrane of both prokaryotic and eukaryotic cells.

Acknowledgments—We are grateful for excellent technical assistance from Geoff Osborne and Sabine Gruninger for help with flow cytometry and Cathy Gillespie for help with confocal microscopy.

REFERENCES

1. Demain, A. L. (1999) *Appl. Microbiol. Biotechnol.* **52,** 455–463
2. Demain, A. L., and Fang, A. (2000) *Adv. Biochem. Eng. Biotechnol.* **69,** 1–39
3. Chai, C. L. L., and Waring, P. (2000) *Redox Rep.* **5,** 257–264
4. Waring, P., Eichner, R. D., and Mullbacher, A. (1988) *Med. Res. Rev.* **8,** 499–524
5. Eichner, R. D., and Mullbacher, A. (1984) *Aust. J. Exp. Biol. Med. Sci.* **62,** 479–484
6. Richard, J. L., Dvorak, T. J., and Ross, P. F. (1996) *Mycopathologia* **134,** 167–170
7. Tomee, J. F., and Kauffman, H. F. (2000) *Clin. Exp. Allergy* **30,** 476–484
8. Mullbacher, A., Moreland, A. F., Waring, P., Sjaarda, A., and Eichner, R. D. (1988) *Transplantation* **46,** 120–125
9. Pahl, H. L., Krauss, B., Schulze-Osthoff, K., Decker, T., Traenckner, E. B., Vogt, M., Myers, C., Parks, T., Waring, P., Mullbacher, A., Czernilofsky, A. P., and Baeuerle, P. A. (1996) *J. Exp. Med.* **183,** 1829–1840
10. Hurne, A. M., Chai, C. L., and Waring, P. (2000) *J. Biol. Chem.* **275,** 25202–25206
11. Waring, P., Sjaarda, A., and Lin, Q. H. (1995) *Biochem. Pharmacol.* **49,** 1195–1201
12. Munday, R. (1982) *Chem. Biol. Interact.* **41,** 361–374
13. Eichner, R. D., Waring, P., Geue, A. M., Braithwaite, A. W., and Mullbacher, A. (1988) *J. Biol. Chem.* **263,** 3772–3777
14. Jordan, T. W., and Pedersen, J. S. (1986) *J. Cell Sci.* **85,** 33–46
15. Eichner, R. D., Al Salami, M., Wood, P. R., and Mullbacher, A. (1986) *Int. J. Immunopharmacol.* **8,** 789–797
16. Waring, P., Newcombe, N., Edel, M., Lin, Q. H., Jiang, H., Sjaarda, A., Piva, T., and Mullbacher, A. (1994) *Toxicon* **32,** 491–504
17. Waring, P., Khan, T., and Sjaarda, A. (1997) *J. Biol. Chem.* **272,** 17929–17936
18. Rice, G. C., Bump, E. A., Shrieve, D. C., Lee, W., and Kovacs, M. (1986) *Cancer Res.* **46,** 6105–6110
19. Ohler, E., Poisel, H., Tataruch, F., and Schmidt, U. (1972) *Chem. Ber.* **105,** 635–641
20. Hansen, M. B., Nielson, S. E., and Berg, K. (1989) *J. Immunol. Methods* **119,** 203–210
21. Ines Borges-Walmsley, M., and Walmsley, A. R. (2000) *Trends Microbiol.* **9,** 71–79
22. Mullbacher, A., Waring, P., Tiwari-Palni, U., and Eichner, R. D. (1986) *Mol. Immunol.* **23,** 231–235
23. Gould, G. W., and Holman, G. D. (1993) *Biochem. J.* **295,** 329–341
24. Brodin, B., Nielsen, C. U., Steffansen, B., and Frokjaer, S. (2002) *Pharmacol. Toxicol.* **90,** 285–296
25. Bernardo, P. H., Chai, C. L., Deeble, G. J., Liu, X. M., and Waring, P. (2001) *Bioorg. Med. Chem. Lett.* **11,** 483–485
26. Rabenstein, D. L., and Weaver, K. (1993) *J. Org. Chem.* **61,** 7391–7397
27. Ernst-Russell, M., Chai, C. L. L., Hurne, A. M., Waring, P., Hockless, D. C. R., and Elix, J. A. (1999) *Aust. J. Chem.* **52,** 279–283
28. Beaver, J. P., and Waring, P. (1995) *Eur. J. Cell Biol.* **68,** 47–54
29. van den Dobbelsteen, D. J., Nobel, C. S., Schlegel, J., Cotgreave, I. A., Orrenius, S., and Slater, F. (1996) *J. Biol. Chem.* **271,** 15420–15427
30. Paz, M. M., Das, A., Palom, Y., He, Q.-Y., and Tomasz, M. (2001) *J. Med. Chem.* **44,** 2834–2842
31. Nagase, T., Kawata, S., Tamura, S., Matsuda, Y., Inui, Y., Yamasaki, E., Ishiguro, H., Ito, T., Miyagawa, J., Mitsui, H., Yamamoto, K., Kinoshita, M., and Matsuzawa, Y. (1997) *Br. J. Cancer* **76,** 1001–1010
32. Moerman, K., Chai, C. L., and Waring, P. (2003) *Toxicol. Appl. Pharmacol.* **190,** 232–240
33. Schweizer, M., and Richter, C. (1994) *Biochemistry* **33,** 13401–13405
34. Perek, N., and Denoyer, D. (2002) *Curr. Drug Metab.* **3,** 97–113
35. Meister, A., and Anderson, M. E. (1997) *Annu. Rev. Biochem.* **52,** 711–760
36. Neilands, J. B. (1995) *J. Biol. Chem.* **270,** 26723–26726
37. Fahey, R. C., Brown, W. C., Adams, W. B., and Worsham, M. B. (1978) *J. Bacteriol.* **133,** 1126–1129